(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number : **0 270 340 B1**

(12) # EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification : **05.10.94 Bulletin 94/40**

(21) Application number : **87310566.2**

(22) Date of filing : **01.12.87**

(51) Int. Cl.[5] : **G01N 33/574,** A61K 49/00, A61K 39/395, A61K 43/00, A61K 47/00, C07K 15/00, G01N 33/577, // C12P21/00, C12N15/00

(54) **Use of an antibody conjugate for the detection of necrotic malignant tissue and for the associated therapy.**

(30) Priority : **05.12.86 US 938425**

(43) Date of publication of application : **08.06.88 Bulletin 88/23**

(45) Publication of the grant of the patent : **05.10.94 Bulletin 94/40**

(84) Designated Contracting States : **AT BE CH DE ES FR GB GR IT LI LU NL SE**

(56) References cited :
CLINICAL RESEARCH, vol. 31, no. 2, 1983; T.G. FULGHUM et al., p. 459A
JOURNAL OF NUCLEAR MEDICINE, vol. 26, no. 5, May 1985; A.M. KEENAN et al., pp. 531-537

(73) Proprietor : **Cancer Biologics, Inc.**
3301 S. Harbor Boulevard
Santa Ana California 92704 (US)

(72) Inventor : **Epstein, Alan L.**
5128 Hillard Avenue
La Canada, CA 91011 (US)
Inventor : **Taylor, Clive R.**
1601 Marengo Avenue
S. Pasadena, CA 91030 (US)

(74) Representative : **Rushton, Ronald et al**
SOMMERVILLE & RUSHTON
11 Holywell Hill
St. Albans Hertfordshire AL1 1EZ (GB)

EP 0 270 340 B1

De cription

I. BACKGROUND OF THE INVENTION

This invention relates to means for rapidly determining the effectiveness of cancer therapy, and to means for augmenting such therapy through the use of antibodies to necrotic or damage neoplastic tissue that are conjugated to labels or pharmaceutically active molecules.

Modern techniques for the nonsurgical treatment of cancer include both clinical and experimental techniques involving chemotherapy, radiation therapy, a combination of chemotherapy and radiation therapy, and immunotherapy. In each instance, the object of the therapy is to kill the malignant cells. Antineoplastic agents presently or potentially useful in such therapy include cytotoxic drugs, biological response modifiers, radiosensitizing compounds, toxins, and radionuclides.

One difficulty associated with cancer therapy is that the effectiveness of a particular therapy varies significantly from one type of cancer to another type of cancer, and even among patients with the same type of cancer. In fact, even individual neoplasms in a single patient may be heterogeneous, having some cells that are more receptive or resistant than others to the particular therapy being utilized. For these reasons, the selection of an effective cancer therapy regimen for a particular patient having a particular type of cancer is not an exact science, but must, in the final analysis, be determined empirically.

A. Monitoring of Effects of Therapy

It is the patient that suffers as a result of this lack of certitude in the establishment of the optimal treatment regimen. The side effects from chemotherapy and radiation therapy are notorious, and include weight loss, vomiting, hearing impairment, hair loss, gastrointestinal damage, and bone marrow damage. Accordingly, physicians make every effort to monitor the effects of the particular treatment regimen being utilized. If the treatment is ineffective, it is discontinued and an alternative treatment is instituted as soon as is feasible.

Conventional methods for monitoring the effectiveness of chemotherapy, radiation therapy, and other nonsurgical cancer therapy include CAT scans, liver-spleen scans, X-rays, Magnetic Resonance (MR) scans, and manual palpation of the tumor, all to detect reduction in tumor size. These techniques are generally useful only after three to four weeks of therapy, since a substantial reduction in tumor size is required in order to identify changes. The patient is therefore committed to a particular therapeutic regimen (and concomitant side effects) until completion of these diagnostic methods.

A monitoring technique that would permit the clinician to determine in a short period of time the effectiveness of a particular therapy in each particular patient would greatly facilitate attainment of the optimum therapeutic regimen while minimizing the time required to do so. Such a technique would also permit the treating physician to minimize the time in which the patient is subjected to ineffective therapy with its accompanying side effects.

B. Augmentation Therapy

Because of the heterogeneous nature of many neoplasms, and because of the mechanisms by which certain therapeutic measures work, not all the cells in a tumor respond to therapy. With a heterogeneous neoplasm, some, but not all of the cells may be susceptible to a particular chemotherapeutic agent. Additionally, radiation therapy and antiproliferative chemotherapeutic agents primarily injure only rapidly growing cells. At any one time, the number of cells in a growth phase is likely to represent only a small number of the total cell population in a tumor. For these reasons, such therapy often reduces, but does not eliminate, the tumor burden. Accordingly, there is a need in that situation for an effective method for destroying the remaining tumor cells.

C. De Novo Therapy of Neoplasms

Finally, because of the hetereogeneity of different types of neoplasms, many different therapeutic approaches have been utilized, forcing clinician and patient to undergo extensive and expensive clinical, radiologic, and laboratory investigations to determine the tumor type. There is therefore a clear need to develop therapeutic approaches applicable in a more uniform way to a broad spectrum of different types of cancer.

Antibodies, and in particular monoclonal antibodies are the focus of intense interest in the field of cancer research. Antibodies hav been d veloped to cell-surface antigens for a number of malignancies, but are useful only in r stricted categories of tumors. Techniques ar known for conjugating such antibodi s to pharmacologically activ agents or to labels to permit diagnosis, localization, and therapy direct d toward such tumors.

Such presently-known conjugates again are useful only in r stricted categories of tumors.

Recent research has led to the identification of unique nuclear antigens and the development of monoclonal antibodies thereto. See, e.g., A. Epstein and C. Clevenger, *Identification of Nuclear Antigens in Human Cells by Immunofluorescence, Immunoelectron Microscopy, and Immunobiochemical Methods Using Monoclonal Antibodies*, Progress in Non-Histone Protein Research, 117 et seq. (I. Bekhor ed. 1985); C. Clevenger and A. Epstein, *Identification of a Nuclear Protein Component of Interchromatin Granules Using a Monoclonal Antibody and* Immunogold Electron Microscopy, Exp. Cell Res. 151: 194-207 (1984); and C. Clevenger and A. Epstein, Use of Immunogold Electron Microscopy and Monclonal Antibodies in the Identification of Nuclear Substructures, J.Histochem. and Cytochem. 32: 757-765 (1984). Such antibodies have been labeled and have been used to identify structures within the nucleus. Id.

The cardiac protein myosin is well known. This protein is an intracellular muscle protein found inside cardiac cells, but not on the cell wall. Myosin-specific antibodies have been developed and have been labeled for in vivo imaging of heart tissue damaged by myocardial infarction. See G. Beller, B. Khaw, E Haber and T Smith, Localization of Radiolabeled Cardiac Myosin-specific Antibody in Myocardial Infarcts, Circulation 55: 74-78 (1977).

Fulghum et al., Clinical Research, 31:459A (1983) discloses that anti-myosin antibodies can be used to detect necrotic myocardial tissues. Such antibodies cannot distinguish between necrotic malignant cells and living tumor cells.

Keenan et al, J Nucl. Med., 26:531-537 (1985) discloses that tumor-associated, or preferably tumor-specific, antibodies are required in order to take clinical advantage of monoclonal antibodies in the treatment and diagnosis of tumors.

II. BRIEF DESCRIPTION OF THE INVENTION

The present invention exploits the observation that antibodies to insoluble intracellular components of cells can be administered in such a way as to show preferential localization to neoplastic cells in vivo, in spite of the known fact that the relevant antigens also are present in normal cells. Such localization is based upon the demonstrated abnormal permeability of a proportion of cancer cells, as well as the specificity and character of the antibody.

In accordance with one aspect of the present invention, there is provided a diagnostic method for measuring effectiveness of therapy intended to kill malignant cells in vivo in a mammal, characterised by the steps of:

obtaining monoclonal antibody that is specific to an insoluble internal cellular component of both normal and malignant cells of said mammal but not to external cellular components, said monoclonal antibody being labeled;

contacting said labeled antibody with tissue removed from said mammal and that has received therapy to kill malignant cells in vivo, thereby permitting said antibody to bind preferentially to said insoluble internal cellular component of cells that have been killed by said therapy; and

determining the effectiveness of said therapy by measuring in vitro, the binding of said labeled antibody to said insoluble internal cellular component in the removed tissue.

Preferred labels include radionuclides, magnetic resonance enhancing agents, and radiopaque materials, and preferred methods for measuring the binding of antibody to antigen include imaging techniques including scintigraphic, magnetic resonance, and radiographic imaging.

In another aspect, the present invention comprises a monoclonal antibody specific to an insoluble intracellular antigen present in neoplastic and normal cells, but not present on the exterior of a living cell, said antibody thus being specific to necrotic tissue but not to living tissue, wherein said antibody is conjugated to a material selected from an alpha-emitting radionuclide, a beta-emitting radionuclide, a radiopaque material, an antineoplastic agent, an immunotherapeutic agent, or a biological response modifier.

The present invention in a further aspect is to a method for preparing an antibody conjugate, comprising the steps of obtaining a monoclonal antibody specific to an insoluble intracellular antigen present in neoplastic and normal cells, but not present on the exterior of a living cell, the antibody thus being specific to necrotic tissue but not to living tissue; and conjugating the antibody to a material selected from an alpha-emitting radionuclide, a beta-emitting radionuclide, a radiopaque material, an antineoplastic agent, an immunotherapeutic agent, or a biological response modifier. The conjugate may be combined with an injectable pharmaceutical carrier.

The present invention also contemplates the use of the antibody conjugated to a radionuclide or a radiopaque material of the present invention in the preparation of a diagnostic composition for imaging neoplastic tissue in vivo in a mammal that has received therapy intended to kill malignant cells in the mammal. It further

includes the use of th antibody conjugated to an antineoplastic agent, an immunotherapeutic agent, or a biological response modifier for preparing a medicament for enhancing in a mammal the effects of antineoplastic therapy that kills malignant cells in vivo.

III. DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Upon cell death and lysis in an an animal, soluble components of the cell, primarily from the cytoplasm, are released. The remainder of the necrotic cell comprises a "cell ghost" made up of various generally insoluble materials that remain "fixed" *in situ* in the tissue. The insoluble cell ghost is gradually destroyed by phagocytosis and enzymatic degradation. At least a portion of the cell ghost remains intact for as long as several weeks. It has been discovered that certain intracellular cell ghost constituents are antigenic. These antigens include nuclear antigens, structural elements, and organelles.

The present invention provides a monoclonal antibody specific to insoluble intracellular antigens for diagnostic and therapeutic purposes, respectively. A diagnostic test in accordance with the present invention for measuring the effectiveness of therapy utilizes such an antibody. The antibody is labeled in a conventional manner. In most instances, the antibodies to insoluble antigens will be labeled with a conventional radiopaque material or a gamma-emitting radionuclide to permit *in vivo* imaging of localized antibody. The same antibody, linked to a pharmacologically-active agent, may be used for therapeutic purposes.

A. Antibody Preparation

The antibodies of the present invention may be obtained through conventional monoclonal antibody preparation techniques. Antigen may be obtained from cells of the species toward which the antibodies are to be directed. For antibodies directed toward human intracellular antigens, malignant cell lines represent a convenient source of such antigen.

Insoluble antigen may conveniently be separated through centrifugation techniques. The cell membranes are disrupted by freezing and thawing, by mechanical techniques, or by other suitable methods. Centrifugation at 1000 x *g* for several minutes is generally sufficient to separate the soluble cytoplasmic fraction from the generally insoluble structural elements and nuclei. Laboratory animals may then be periodically immunized according to accepted procedure to generate the desired immunologic response.

To generate monoclonal antibodies, murine spleen cells from immunized animals are fused with an appropriate myeloma cell line. Fused cells are cultured in selective growth medium, and culture supernatants from active cell cultures are tested for antibody activity. Positive cultures are identified and expanded.

In order to screen for monoclonal antibodies that bind specifically to cell ghosts with little or no binding to live cells, equal aliquots of normal and neoplastic live cells are prepared. To obtain cell ghosts, one aliquot each of neoplastic and normal cells is subjected to several rapid freeze-thaw cycles, and is then washed with buffer to remove soluble components. The ability of monoclonal antibody from each tested culture to bind, respectively, the cell ghosts and the intact cells is then quantitatively measured. One appropriate measurement technique is a radioimmunoassay. Thus, when using murine monoclonal antibody, radiolabeled anti-mouse IgG may be used to quantitate the amount of bound mouse antibody. Direct or indirect immunofluorescence screening techniques may also be used. Specificity for insoluble intracellular antigens may be determined by comparing the amount of antibody bound to cell ghosts with that bound to intact cells.

Antibodies to intracellular antigen identified above are then screened to ensure there is no binding to soluble antigen obtained from necrotic cultures of normal and neoplastic cells by measuring the binding of the antibody in question to the supernatant from those necrotic cultures. Conventional radioimmunoassay techniques may be used.

B. Labels for Antibodies

(1) Radiolabels

For imaging purposes, any of the well-known medical radionuclides can be used. Suitable radionuclides include Tc-99m, I-123, I-125, I-111, In-113m, Ga-67, or other suitable gamma-emitters.

(2) Radiopaque Materials

Radiopaque materials also may be used to label the antibodi s. Suitable radiopaque materials are well known and include iodin compounds, barium compounds, gallium compounds, thallium compounds, and the

like. Specific examples of radiopaque materials include barium, diatrizoate, ethiodized oil, gallium citrate, iocarmic acid, iocetamic acid, iodamid , iodipamide, iodoxamic acid, iogulamide, iohexol, iopamidol, iopanoic acid, ioprocemic acid, iosefamic acid, ioseric acid, iosulamide meglumine, iosumetic acid, iotasul, iotetric acid, iothalamic acid, iotroxic acid, ioxaglic acid, ioxotrizoic acid, ipodate, meglumine, metrizamide, metrizoate, propyliodone, and thallous chloride.

(3) Magnetic Resonance - Enhancing Materials

Materials that can be detected by or that enhance the effects of magnetic resonance imaging equipment also may be conjugated to the antibodies. Suitable conventional magnetic resonance-enhancing compounds include gadolinium, copper, iron, and chromium. It is preferred that these metal atoms be prepared in the form of a conventional organometallic chelates, which are then bound to the antibody.

C. Therapeutic Agents

A large number of antineoplastic agents are known, many of which can be conjugated to the antibodies of the present invention using known techniques. These antineoplastic agents may include folate inhibitors, pyrimidine analogs, purine analogs, alkylating agents, antibiotics, and radiosensitizing compounds. Specific examples of such antineoplastic agents include acivicin, aclarubicin, acodazole, adriamycin, ametantrone, aminoglutethimide, anthramycin, asparaginase, azacitidine, azetepa, bisantrene, bleomycin, busulfan, cactinomycin, calusterone, caracemide, carboplatin, carmustine, carubicin, chlorambucil, cisplatin, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin, dezaguanine, diaziquone, doxorubicin, epipropidine, etopside, etoprine, floxuridine, fludarabine, fluorouracil, fluorocitabine, hydroxyurea, iproplatin, leuprolide acetate, lomustine, mechlorethamine, megestrol acetate, melengestrol acetate, mercaptopurine, methotrexate, metoprine, mitocromin, mitogillin, mitomycin, mitosper, mitoxantrone, mycophenolic acid, nocodazole, nogalamycin, oxisuran, peliomycin, pentamustine, porfiromycin, prednimustine, procarbazine hydrochloride, puromycin, pyrazofurin, riboprine, semustine, sparsomycin, spirogermanium, spiromustine, spiroplatin, streptozocin, talisomycin, tegafur, teniposide, teroxirone, thiamiprine, thioguanine, tiazofurin, triciribine phosphate, triethylenemelamine, trimetrexate, uracil mustard, uredepa, vinblastine, vincristine, vindesine, vinepidine, vinrosidine, vinzolidine, zinostatin and zorubicin.

In addition, alpha-emitting and beta-emitting radionuclides may be used. Such compounds include I-131, Yt-99, Cu-67, Au-198, P-32, and other cytotoxic radionuclides.

The antibodies of the present invention also may be conjugated to biological response modifiers, including interleukin-2, vasodilators, any of the interferons, tumor necrosis factor, and the like.

Yet another category of compounds that may be bound to the antibodies of the present invention are toxins such as ricin, tetanus, diptheria, abrin, gelonin, mistletoe, and other materials capable of causing localized necrosis.

D. Conjugation of Labels and Therapeutic Compounds to Antibodies

Numerous techniques suitable for binding various molecules to antibodies have been established. Iodination, for example, may be accomplished using the chloramine-T method described by S. Mills, et al., *^{123}I-Radiolabeling of Monoclonal Antibodies for In Vivo Procedures,* Hybridoma 5: 265-275 (1986). This technique may be used to effect iodination to render the antibody radiopaque, or to attach a radionuclide, such as I-125 or I-131.

Other radionuclides may be attached to the antibodies in question through chelation with benzyl EDTA or DPTA conjugation procedures. Still other suitable techniques include the iodogen method disclosed by M. Pimm, et al., *In Vivo Localization of Anti-Osteogenic Sarcoma 791T Monoclonal Antibody,* Int. J. Cancer 30: 75 (1982), and direct iodination with radioactive sodium iodide.

Numerous techniques are available for attaching various molecules, enzymes and proteins to antibodies. For example, many carboxylic acid-containing compounds (such as methotrexate) can be conjugated to immunoglobulins through an active ester intermediate, formed, e.g., by reacting the compound with N-hydroxysuccinimide and dicyclohexylcarbodiimide. See, T. Deguchi, et al., *Effect of Methotrexate-Monoclonal Anti-Prostatic Acid Phosphatase Antibody Conjugate on Human Prostate Tumor,* Cancer Res. 46: 3751-3755 (1986). Others, such as chlorambucil, will bind directly to the antibodies at low pH. See, e.g., T. Ghose, et al., *Immunochemotherapy of Human Malignant Melanoma with Chloroambucil-Carrying Antibody,* Europ. J. Cancer 11: 321-326 (1975).

Amino sugar-containing drugs such as adriamycin and daunomycin may be covalently bound to antibodies

by periodate oxidation of the drug, followed by linking of the oxidized drug to the immunoglobulin and subsequent reduction of the product with sodium borohydride. E. Hurwitz, et al., *The Covalent Binding of Daunomycin and Adriamycin to Antibodies*, Cancer Res. 35: 1175-1181 (1975).

Conventional techniques also exist for binding biological response modifiers or other proteins to antibodies. Free thiol groups may be introduced into the antibody by reacting antibody with N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) to introduce 2-pyridyl disulphide groups, which are reduced with dithiotreitol to leave free thiol groups. The protein to be bound to the antibody is incubated with SPDP. Upon mixing the SPDP-modified protein with the antibody containing free thiol groups, the two materials become bound.

Other known techniques, such as the use of dextran T-10 spacers to increase the number of drug moieties linked to antibody molecules can be employed, as can mixed anhydride methods of drug conjugation. The compound 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (ECDI) may be used to bind amino-containing drugs to the carboxyl groups of antibodies. Alternatively, glutaraldehyde may be used for cross-linking between free amino groups of the antibody and amino groups in the compound to be conjugated thereto.

E. Monitoring and Diagnostic Procedures Using Antibodies to Insoluble Intracellular Antigens

One diagnostic or monitoring procedure uses labeled antibodies to insoluble intracellular antigens, wherein the label is a gamma-emitting radionuclide of the type previously discussed. This labeled antibody is injected (preferably intravenously) into a patient who has received chemotherapy, radiation therapy, or both. This procedure is preferably carried out at least one or two days after the initiation of the therapy, in order to permit resultant necrosis of the neoplastic tissue to advance to a sufficient point that reasonable numbers of cell ghosts are present. Between 30 minutes and 3 days following administration of the labeled antibody, and appropriate scintigraphic imaging technique is employed to image label localized in necrotic tissue. Suitable imaging techniques include gamma cameras and SPECT (single photon emission computed tomography) techniques.

One alternative imaging technique is radiographic imaging. In this technique, antibody to insoluble intracellular antigen that has been labeled with a radiopaque material is injected a suitable time after initiation of chemotherapy or radiation therapy. After the antibody has localized at the areas of necrotic tissue, radiographic imaging is performed. Other suitable techniques include CAT (computed axial tomography) scans, fluoroscopy and conventional X-ray imaging.

F. Therapeutic Procedures Using Antibodies to Insoluble Intracellular Antigens

By conjugating the antibodies of the present invention to therapeutic, antineoplastic compounds, those therapeutic agents may be delivered directly to the neoplasm, with greatly reduced systemic effect. Two approaches may be employed; first, as augmentation therapy following use of an existing therapeutic modality that may have killed all or part of the tumor; and second, as a primary de *novo* mode of therapy focusing on the neoplastic cells already showing some degree of abnormal permeability for the therapeutic antibody.

In the augmentation approach, tumor necrosis is initiated by any conventional technique, such as chemotherapy, immunotherapy, radiation therapy, or the like. After initiation of such therapy, necrosis begins and cell ghosts appear in the tumor mass. At this point (usually at least two days after initiation of the primary therapy), a conjugate of a antibody to an insoluble intracellular antigen and a therapeutic compound, preferably an antineoplastic agent, is administered to the patient. Intravenous administration is preferred, although direct injection in the vicinity of the tumor is also contemplated.

Following administration of the antineoplastic agent-antibody conjugate, antibody becomes bound to the cell ghosts in the tumor mass, bringing with it the antineoplastic agent.

If a lethal amount of immunoconjugate is delivered to a necrotic area, healthy, viable tumor cells surrounding this area may be rendered necrotic, enabling additional amount of immunoconjugate to penetrate the newly necrotic area. In this way, a gangrene-like effect may be possible with destruction of the tumor cells proceeding radially from necrotic to healthy tumor tissue. To achieve this gangrene-like effect, a cytotoxic agent such as a beta-emitting or an alpha-emitting radionuclide may be used.

In the second approach (primary therapy), the method is exactly as described above, except that the need for prior treatment with some other modality (to create an initial population of necrotic cells) is obviated.

The amount of antineoplastic agent-antibody conjugate administered to the patient will vary depending on the antineoplastic agent used and the size of the tumor. However, in general, the dosage is selected to administer a total dose of antineoplastic agent that is equal to or less than the conventional th rapeutic dosage of the particular agent select d. It is preferred that the total dosag be between 1% and 50% of th conventional therapeutic dosage, and it is most preferred that the dosage be between 2% and 25% of th conventional ther-

apeutic dosage of th compound. However, as with all cancer therapy, the optimum dosage will b determined by the treating physician based on the individual patient's respons to the therapy and the side effects resulting therefrom.

EXAMPLE 1

In order to generate hybridomas producing monoclonal antibody to nuclear antigens, eight human malignant lymphoma and leukemia cell lines were used as a source of antigens. These include the EBV-positive nonproducer Raji and producer AG876 African Burkitt's lymphoma cell lines; the T-cell acute lymphoblastic leukemia CEM cell line; the IgE secreting multiple myeloma U-266 cell line; the erythroleukemia K562 cell line; and the histiocytic type SU-DHL-1 and U-937 and B-cell type SU-DHL-4 diffuse histiocytic lymphoma cell lines. In addition to these cultures, normal peripheral blood lymphocytes pooled from several individuals and separated by the ficoll-hypaque technique were used alone and after four days of stimulation with 5ug/ml of Pokeweed mitogen. In order to help characterize and screen the monoclonal antibodies, HeLa cells and a normal diploid human fibroblast cell strain established from a skin punch biopsy were used in the immunofluorescence and immunoelectron microscopy experiments. All of the cultures were grown in RPMI-1640 medium (GIBCO) supplemented with 15% fetal calf serum, 100 units/ml Penicillin-G an 100 ug/ml streptomycin sulfate. In order to produce large quantities of cells, 3 liter hanging bar spinner flasks were used after seeding with approximately 4 x 10^8 cells. The cells were harvested 4 to 5 days later, washed twice in phosphate buffered saline (PBS), resuspended in 10 ml of buffer A (150 m*M* NaCl, 20 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* 2-mercaptoethanol, 0.5 m*M* phenylmethylsulfonylfluoride and 1% aprotinin) containing 10% glycerol, and frozen at -85°C for future use. Generally, 3 liters of cells yielded 2.5 ml of packed cells per harvest.

In order to obtain nuclear extracts from the human malignant lymphoma and leukemia cell lines and peripheral blood lymphocytes, 2.5 ml of packed cells were thawed and washed once with Ca/pipes buffer (0.01 *M* $CaCl_2$, 2 x 10^{-3} *M* piperazine-N, N^1-bis (2-ethanesulfonic acid)) in a 50-ml centrifuge tube. All procedures were performed at 4°C and centrifugation was at 1000 x *g* for 10 minutes. The pellet was then resuspended in 40 ml of Ca/pipers buffer and thoroughly homogenized using a motor-driven polytetrafluoroethylene pestle to disrupt the swollen cells. The nuclei were then pelleted leaving a cloudy supernatant as the cytoplasmic fraction and resuspended in Ca/pipes buffer containing 1% NP-40 (Gallard-Schlesinger). The nuclei were then rehomogenized to remove nuclear membranes and checked by phase-contrast microscopy to be free of contaminating cytoplasmic and membranous debris. Nuclei were then washed twice in Ca/pipes buffer to remove the detergents, resuspended in 8 ml of phosphate-buffered saline, and sonicated three times for 15 seconds at 30-second intervals in order to produce a more homogeneous suspension. The resulting nuclear extracts, which were free of major cytoplasmic proteins, were then frozen in 1-ml aliquots at -85°C for use in the immunization procedures.

One-milliliter aliquots of the nuclear extracts from each of the eight human lymphoma and leukemia cell lines and the peripheral blood lymphocytes were thawed, resonicated to reduce viscosity, and emulsified in 1.5 ml of complete Freund's adjuvant using two glass syringes and a 20-gauge microemulsifying needle. Three 8-week-old Balb/c female mice per sample were injected subcutaneously at multiple sites using a 22-gauge needle and glass syringe. The mice were reinoculated 2 weeks later as above except the nuclear extracts were prepared in incomplete adjuvant.

The mice received a third inoculation of antigen 10 days later, this time without adjuvant and by intraperitoneal injection. The mice were sacrificed by cervical dislocation 4 days later and the spleens removed using aseptic technique for the hybridoma experiments.

Spleen cells from the sacrificed Balb/c mice were fused with 8-azaguanine resistant mouse myeloma NS-1 cells at a ratio of 5:1, respectively, using 40% polyethylene glycol (PEG) having a molecular weight of 1540. After fusion, the cells were cultured in selective HAT medium containing 10^{-4} *M* hypoxanthine, 4 x 10^{-7} *M* aminopterin, and 1.5 x 10^{-5} *M* thymidine in a 96-well microtiter plate at a concentration of 2 x 10^5 cells per well. The medium was changed every 3 days and, after the first week, the aminopterin was discontinued. Culture supernatants from wells with active cell growth were tested by indirect immunofluorescence for antibody activity. To help harvest the supernatants, a multichannel pipette was used to transfer the supernatants to other 96-well plates. In order to prevent cross-contamination from well to well, a new sterile pipette tip was used for each supernatant fluid. Positive cultures were transferred to a 24-well cluster plate for expansion and an aliquot of cells was cloned on 0.5% Noble agar containing RPMI-1640 medium, 20% fetal calf serum, and antibiotics. Agar plates were prepared in 60 x 15 mm tissue culture petri dishes the day before cloning and were stored at 4°C until us . In order to assure the growth of an adequate number of colonies, plates were seeded with 500 or 1000 cells in a volume of 50 ul. The cells wer then spread on the surface of the agar with a glass rod bent at 90° and the plat s were incubated undisturbed for 10 to 12 days in a well-humidified 5% CO_2 incubator

at 37°C. AT this time, a maximum of 24 colonies per hybridoma wer picked with Past ur pipettes and transferred to a 96-well plate for continued growth. Supernatants of these cultures were then retested 4 to 5 days later when the media turned acidic. Positive cultures were then expanded slowly and, when actively growing in tissue culture flasks, they were frozen in liquid nitrogen for safe storage in RPMI-1640 medium containing 20% fetal calf serum, antibiotics and 10% dimethylsulfoxide (DMSO) at a concentration of 5×10^6 cells per milliliter.

EXAMPLE 2

Antibody for screening was selected from a library of monoclonal antibodies to intracellular antigens that includes the antibodies produced by the hybridomas of Example 1. The selected antibody was screened as follows:

Large cell lymphoma cells (SU-DHL-2) and adenocarcinoma lung cancer cells (A549) were divided into two equal aliquots. One aliquot was washed 2 times with PBS containing 1 mg/ml bovine serum albumin and 0.02% sodium buffer (wash buffer) and placed in 4 ml tubes at a concentration of 1×10^6 cells/tube. The other aliquot was freeze-thawed 3 times rapidly and washed 3 times with wash buffer to remove soluble components. The cell ghosts were then equally divided into the same number of 4 ml tubes as the first aliquots. One tube from each set was then incubated with 1 ml of monoclonal antibody supernatant for 1 hour with continuous shaking at room temperature. The cells were washed 2 times with wash buffer to remove unbound antibody and then incubated with 100,000 cpm of I-125 goat anti-mouse IgG radiolabeled probe to quantitate the amount of bound mouse immunoglobulin. After a 1 hour incubation with continuous shaking a room temperature, the cells were washed 3 times with wash buffer and counted in a gamma counter for 1-minute intervals.

The purpose of this radioimmunoassay was to identify among a subset of antinuclear monoclonal antibodies those reagents which bind specifically to dead cells with little or no binding to live viable cells. A lymphoma cell line and lung cancer cell line were chosen to show that certain antibodies may be used against a wide variety of human tumors to bind and identify dead cells. Three candidate antibodies, 877-8, 898-9 and 899-4, were identified through this limited screening. The results of this procedure are shown in Table 1.

TABLE 1.

Data are expressed as counts per minute (CPM)
High counts denote antibody binding

Monoclonal Antibody	SU-DHL-2		A549	
	Live Cells	Dead Cells	Live Cells	Dead Cells
244-7	5,934	6,772	3,426	5,509
364-5	769	622	656	1,036
372-2	1,592	437	2,024	1,896
443-4	1,211	919	560	1,337
652-2	6,019	1,355	11,697	9,176
780-3	557	1,063	3,163	1,205
785-5	746	648	1,211	2,197
841-19	1,645	1,851	3,478	2,517
859-4	327	1,504	1,974	3,604
* 877-8	1,481	1,833	1,641	4,680
891-5	1,247	2,834	2,856	5,973
* 898-9	3,442	2,726	2,317	13,942
* 899-4	1,980	8,193	2,232	3,534
1415-1	5,550	6,158	3,821	6,363
1702-5	4,711	1,786	4,696	2,666
NS-1 (neg. control)	552	507	346	738

In order to understand the significance of the data in Table 1, it is important to realize that even a "live " *in vitro* culture will contain a relatively large proportion of necrotic cells, as opposed to a population of similar cells *in vivo*. Thus, some binding to the "live" cell culture can be expected, even with an antibody that is specific to only insoluble intracellular antigen. It should also be recognized that even though the antibody may not be specific to any surface protein or antigen of the cell line employed in the screening process, certain tumor cell lines (such as histiocytic cell lines) have surface components that exhibit generalized binding of immunoglobulins.

Prior to commencing animal trials, the candidate antibodies identified by the foregoing screening procedure are further screened against normal live and dead cells to confirm that the antibodies are specific to insoluble intracellular antigen found in all cells in the mammal in which the antibodies will be used. Further, the antibodies are screened against the supernatant of necrotic cells to confirm that the antibody does not bind to soluble intracellular antigen.

EXAMPLE 3

Antibody is identified in accordance with the procedure of Example 2 that is specific to insoluble intracellular antigen not present on the cell surface of normal or malignant cells.

In order to reduce the size of the eventual antibody conjugate, purified monoclonal antibody is subjected to pepsin digestion for 20 hours at 37°C to prepare $(Fab')_2$ fragments. These $(Fab')_2$ fragments are then separated from intact antibody and protein aggregates through Sephadex G-100 (90 x 2.5 cm) column chromotography. The first protein peak to be eluted contains the $(Fab')_2$ component of the antibody.

The antibody is label d with I-131 through th method of S. Mills, et al., *supra*. Using this iodination procedure, and averag of at least one or more atoms of I-131 is conjugated to each molecule of monoclonal antibody.

Conventional chemotherapy is initiated for a patient suffering from a solid tumor. Appropriate ch motherapy is administered intravenously. After 24 hours, 1 mg of monoclonal antibody labeled with 10 miCi I-131 is administered intravenously in a 10 ml sterile saline suspension. Six hours laters, the patient is imaged with a gamma camera, which shows strong localized binding of the radiolabeled antibody to the tumor site. The intensity of tumor imaging, when compared with images obtained under identical conditions prior to therapy, is indicative of the degree to which the chemotherapy has resulted in tumor cell death. In addition, it permits visualization of the primary tumor, and localization of metastases of significant size.

Forty-eight hours after the initiation of chemotherapy, chlorambucil antibody conjugate to insoluble intracellular antigen (using the $(Fab')_2$ fragment) is administered intravenously. The antibody-drug conjugate localizes to the cell ghosts of the tumor, delivering the chemotherapeutic alkylating agent to the site of the tumor, with therapeutic effect.

Claims

1. A method for preparing an antibody conjugate, comprising the steps of:
   obtaining a monoclonal antibody to an insoluble intracellular antigen present in neoplastic and normal cells, but not present on the exterior of a living cell, said antibody thus being specific to necrotic tissue not to living tissue; and
   conjugating said antibody to a material selected form an alpha-emitting radionuclide, a beta-emitting radionuclide, a radiopaque material, an antineoplastic agent, an immunotherapeutic agent, or a biological response modifier.

2. The method of Claim 1, wherein said material is an antineoplastic agent.

3. A method of Claim 1, wherein said material is a radionuclide.

4. The method of any of Claims 1-3, further comprising the step of combining the conjugate with an injectable pharmaceutical carrier.

5. A monoclonal antibody to an insoluble intracellular antigen present in neoplastic and normal cells, but not present on the exterior of a living cell, said antibody thus being specific to necrotic tissue but not to living tissue, wherein said antibody is conjugated to a material selected from an alpha-emitting radionuclide, a beta-emitting radionuclide, a radiopaque material, an antineoplastic agent, an immunotherapeutic agent, or a biological response modifier.

6. The antibody of Claim 5, wherein said antibody is conjugated to a radionuclide.

7. The antibody of Claim 5, wherein said antibody is conjugated to a radiopaque material.

8. The antibody of Claim 5, wherein said antibody is conjugated to an antineoplastic agent, an immunotherapeutic agent, or a biological response modifier.

9. The antibody of any of Claims 5-8, in combination with an injectable pharmaceutical carrier.

10. Use of the antibody conjugate of Claim 6 or 7 in the preparation of a diagnostic composition for imaging neoplastic tissue in vivo in a mammal that has received therapy intended to kill malignant cells in said mammal.

11. Use of the antibody-antineoplastic agent conjugate of Claim 8 in the preparation of a medicament for enhancing in a mammal the effects of therapy that kills malignant cells in vivo.

12. A use as claimed in Claim 11, wherein said antineoplastic agent comprises at least one of a cytotoxic agent, a toxin, a biological response modifier, a radiosensitising compound, an alpha-emitting radionuclide, a beta-emitting radionuclide, and an antiproliferative agent.

13. A diagnostic method for measuring effectiveness of therapy intended to kill malignant cells in vivo in a mammal, characterised by the steps of:
   obtaining monoclonal antibody that is specific to an insoluble internal cellular component of both normal and malignant cells of said mammal but not to external cellular components, said monoclonal an-

tibody being labeled;

contacting said labeled antibody with tissue removed from said mammal and that has received therapy to kill malignant cells in vivo, thereby permitting said antibody to bind preferentially to said insoluble internal cellular component of cells that have been killed by said therapy; and

determining the effectiveness of said therapy by measuring in vitro, the binding of said labeled antibody to said insoluble internal cellular component in the removed tissue.

14. The method of Claim 13, wherein said label is a radionuclide.

15. The method of Claim 13, wherein said label is a radiopaque material.

Patentansprüche

1. Verfahren zur Herstellung eines Antikörper-Konjugats, umfassend folgende Stufen:

Gewinnen eines monoklonalen Antikörpers, der für ein in neoplastischen und normalen Zellen vorhandenes, unlösliches, intrazelluläres Antigen spezifisch ist, aber nicht außen an einer lebenden Zelle vorhanden ist, wobei der Antikörper spezifisch gegen nekrotisches, aber nicht für lebendes Gewebe ist;

Konjungieren des Antikörpers mit einem Material, ausgewählt unter: ein α-emittierendes Radionuklid, ein β-emittierendes Radionuklid, ein strahlenundurchlässiges Material, ein antineoplastisches Mittel, ein immunotherapeutisches Mittel oder ein Modifikator der biologischen Reaktion.

2. Verfahren nach Anspruch 1, wobei es sich bei dem Material um ein antineoplastisches Mittel handelt.

3. Verfahren nach Anspruch 1, wobei es sich bei dem Material um ein Radionuklid handelt.

4. Verfahren nach einem der Ansprüche 1 bis 3, ferner umfassend die Stufe der Kombination des Konjugats mit einem injizierbaren pharmazeutischen Träger.

5. Monoklonaler Antikörper, der gegen ein unlösliches, intrazelluläres Antigen, das in neoplastischen und normalen Zellen vorhanden ist, jedoch nicht außen an einer lebenden Zelle vorhanden ist, spezifisch ist, wobei der Antikörper somit gegen nekrotisches, aber nicht gegen lebendes Gewebe spezifisch ist, wobei der Antikörper mit einem Material konjugiert ist, das ausgewählt ist unter: ein α-emittierendes Radionuklid, ein β-emittierendes Radionuklid, ein strahlenundurchlässiges Material, ein antineoplastisches Mittel, ein immunotherapeutisches Mittel oder ein Modifikator der biologischen Reaktion.

6. Antikörper nach Anspruch 5, wobei der Antikörper mit einem Radionuklid konjugiert ist.

7. Antikörper nach Anspruch 5, wobei der Antikörper mit einem strahlenundurchlässigen Material konjugiert ist.

8. Antikörper nach Anspruch 5, wobei der Antikörper mit einem antineoplastischen Mittel, einem immunotherapeutischen Mittel oder mit einem Modifikator der biologischen Reaktion konjugiert ist.

9. Antikörper nach einem der Ansprüche 5 bis 8 in Kombination mit einem injizierbaren pharmazeutischen Träger.

10. Verwendung des Antikörper-Konjugats nach Anspruch 6 oder 7 bei der Herstellung einer diagnostischen Zusammensetzung zur in vivo-Abbildung von neoplastischem Gewebe in einem Säuger, der einer zur Abtötung von malignen Zellen in Säuger vorgesehenen Therapie unterzogen worden ist.

11. Verwendung des Antikörper-antineoplastisches Mittel-Konjugats nach Anspruch 8, bei der Herstellung eines Arzneimittels zur Verstärkung der Wirkung der Therapie, die in vivo maligne Zellen abtötet, bei einem Säuger.

12. Verwendung nach Anspruch 11, wobei das antineoplastische Mittel mindestens einen der folgenden Bestandteile umfaßt: ein zytotoxisches Mittel, ein Toxin, ein Modifikator der biologischen Reaktion, eine strahlungssensibilisierende Verbindung, ein α- mittierendes Radionuklid, ein β-emittierendes Radionuklid und antiproliferatives Mittel.

13. Diagnostisches Verfahren zur Messung der Wirksamkeit ein r Therapie, die bei inem Säuger zur in vivo-Abtötung von malignen Zellen vorgesehen ist, gekennzeichnet durch folgende Stufen:

Gewinnen eines monoklonalen Antikörpers, der gegen eine unlösliche, interne, zelluläre Komponente von normalen und malignen Zellen dieses Säugers, jedoch nicht gegen externe zelluläre Komponenten spezifisch ist, wobei der monoklonale Körper markiert ist;

Kontaktieren des markierten Antikörpers mit Gewebe, das dem Säuger entfernt worden ist und das einer Therapie zur in vivo-Abtötung von malignen Zellen unterzogen worden ist, wobei man den Antikörper bevorzugt eine Bindung mit der unlöslichen, internen, zellulären Komponente der Zelle, die durch die Therapie abgetötet worden sind, eingehen läßt, ;

Bestimmen der Wirksamkeit dieser Therapie durch die in vitro-Messung der Bindung des markierten Antikörpers an die unlösliche, interne, zelluläre Komponente im entfernten Gewebe.

14. Verfahren nach Anspruch 13, wobei es sich bei der Markierung um ein Radionuklid handelt.

15. Verfahren nach Anspruch 13, wobei es sich bei der Markierung um ein strahlenundurchlässiges Material handelt.

Revendications

1. Procédé de préparation d'un conjugué d'anticorps, comprenant les étapes consistant à :

obtenir un anticorps monoclonal spécifique d'un antigène intracellulaire insoluble présent dans des cellules néoplasiques et normales, mais non présent sur l'extérieur d'une cellule vivante, ledit anticorps étant ainsi spécifique d'un tissu nécrotique mais pas d'un tissu vivant, et

conjuguer ledit anticorps à une matière choisie parmi un radionucléide émetteur alpha, un radionucléide émetteur bêta, une matière opaque à la radiographie, un agent antinéoplasique, un agent immunothérapeutique ou un modificateur de réponse biologique.

2. Procédé selon la revendication 1, dans lequel ladite matière est un agent antinéoplasique.

3. Procédé selon la revendication 1, dans lequel ladite matière est un radionucléide.

4. Procédé selon l'une des revendications 1 à 3, comprenant en outre l'étape de combinaison du conjugué avec un véhicule pharmaceutique injectable.

5. Anticorps monoclonal spécifique d'un antigène intracellulaire insoluble présent dans des cellules néoplasiques et normales, mais non présent sur l'extérieur d'une cellule vivante, ledit anticorps étant ainsi spécifique d'un tissu nécrotique mais pas d'un tissu vivant, ledit anticorps étant conjugué à une matière choisie parmi un radionucléide émetteur alpha, un radionucléide émetteur bêta, une matière opaque à la radiographie, un agent antinéoplasique, un agent immunothérapeutique ou un modificateur de réponse biologique.

6. Anticorps selon la revendication 5, ledit anticorps étant conjugué à un radionucléide.

7. Anticorps selon la revendication 5, ledit anticorps étant conjugué à une matière opaque à la radiographie.

8. Anticorps selon la revendication 5, ledit anticorps étant conjugué à un agent antinéoplasique, un agent immunothérapeutique ou un modificateur de réponse biologique.

9. Anticorps selon l'une des revendications 5 à 8, en combinaison avec un véhicule pharmaceutique injectable.

10. Utilisation de l'anticorps conjugué selon la revendication 6 ou 7 dans la préparation d'une composition diagnostique pour la formation d'images de tissus néoplasiques "in vivo" d'un mammifère qui a été soumis à une thérapie destinée à tuer des cellules malignes dans ce mammifère.

11. Utilisation de l'anticorps conjugué à un agent antinéoplasique selon la revendication 8 dans la préparation d'un médicament pour augmenter, chez un mammifère, les effets d'une thérapie qui tue des cellules malignes "in vivo".

12. Utilisation selon la revendication 11, dans laquelle ledit agent antinéoplasique comporte au moins l'un des agents suivants : un agent cytotoxique, une toxine, un modificateur de réponse biologique, un composé radiosensibilisateur, un radionucléide émetteur alpha, un radionucléide émetteur bêta et un agent d'antiprolifération.

13. Procédé diagnostique pour mesurer l'efficacité d'une thérapie destinée à tuer des cellules malignes "in vivo" chez un mammifère, caractérisé par les étapes consistant à :

obtenir un anticorps monoclonal qui est spécifique d'un composant cellulaire interne insoluble de cellules normales et de cellules malignes dudit mammifère, mais pas spécifique de composants cellulaires externes, ledit anticorps monoclonal étant marqué;

mettre ledit anticorps marqué en contact avec un tissu prélevé sur ledit mammifère et ayant été soumis à une thérapie pour tuer des cellules malignes "in vivo", en permettant ainsi audit anticorps de se lier de manière préférentielle audit composant cellulaire interne insoluble de cellules qui ont été tuées par ladite thérapie; et

déterminer l'efficacité de ladite thérapie par mesure "in vitro" de la liaison entre ledit anticorps marqué et ledit composant cellulaire interne insoluble dans le tissu prélevé.

14. Procédé selon la revendication 13, dans lequel la marque est un radionucléide .

15. Procédé selon la revendication 13, dans lequel la marque est une matière opaque à la radiographie.